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Retention of different-sized particles and derived gut fill estimate in tammar wallabies
(*Macropus eugenii*): physiological and methodological considerations

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Abstract

The capacity of the digestive tract is an important parameter in understanding digestive adaptations, particularly in herbivores. Measures of this capacity ('gut fill') are commonly performed in killed animals, which has ethical and logistical implications. Alternatively, dry matter gut contents (DMC) can be estimated in live animals from food intake, digesta retention and digestibility, based on physical principles (Holleman and White 1989). Although this method has been used to some extent, it still awaits thorough validation. Here we estimated DMC in seven tammar wallabies during 5-day feeding trials and compared the results to those gained from dissections immediately after the trials. Calculated DMC exceeded that actually measured by $29 \pm 22 \%$. A closer inspection of the data suggested that this is partly due to the fact that DMC as measured by dissection is susceptible to short-term influences such as daily variation in food intake, whereas the calculated DMC represents an integrative measure over the whole period of the feeding trial. Correlations between both the measured digesta retention times, and the calculated DMC, with the measured wet contents mass suggest that it is particularly the DMC determined via dissection that needs to be measured with care. For a comparison of gut capacities, the calculated DMC therefore can be considered adequate, but should for a more widespread use be validated in further studies including more species and experimental regimes controlling food intake variation.

Additionally, we tested whether very small (100-500 μm) and small (500-1000 μm) particles were retained differently in the tammar wallabies. There was no indication of such a difference. Whether the macropod forestomach selectively passes a certain particle fraction (that represents microbes) with the generally faster-passing fluids remains to be investigated with even smaller markers, e.g. labelled bacteria.

Keywords: herbivory, digestion, intake, retention, gut capacity, macropod

Introduction

The capacity of the digestive tract is one important parameter in the digestive adaptations of animals (Hume 2005). Differences in this capacity may change with age, reproductive status, food quality, intake level, and species. Conventionally, gut capacity is measured by gravimetry or volumetry of full or empty sections of the digestive tract (Clauss et al. 2007). This requires killing of animals, which has ethical implications, may not always be possible in every trial setting, and evidently precludes repeated measurements on individuals under different conditions.

Holleman & White (1989) proposed a method of estimating gut fill in live animals by recording, in a steady state, food intake, digestibility, and digesta retention time, based on simple physical principles. A corresponding approach had already been demonstrated by Blaxter et al. (1956). This method has been applied in various studies to test for inter- and intraspecific differences in gut capacity (Baker and Hobbs 1987; Gross et al. 1996; Behrend et al. 2004; Munn and Dawson 2006; Munn and Barboza 2008; Schwarm et al. 2009c; Clauss et al. 2010b; Franz et al. 2011; Sawada et al. 2011). However, although the underlying principle of the derivation is logical, doubts on the reliability of the approach might still prevail as long as the method has not been thoroughly validated. In the original study, Holleman & White (1989) limited the validation to a sample size of five sheep, and found that the calculated gut fill was only 3.3 % lower than the actually measured one. Here, we expanded this validation to seven individuals of a smaller, marsupial herbivore species, the tammar wallaby (*Macropus eugenii*).

One important question that was not addressed in Holleman and White's (1989) original study is which particle-size marker should be used to estimate digesta retention times.

Especially in ruminants, different-sized particles will be retained for different periods in the digestive tract (Schwarm et al. 2008; Schwarm et al. 2009a; Lechner et al. 2010; Clauss et al. 2011). The choice of the particle-size marker will thus evidently influence the derived gut fill estimate. For example, given a mean dry matter intake of 3.4 kg d^{-1} with a dry matter digestibility of 56 % and a mean retention time of 51 h for 2 mm-particles and 58 h for 10 mm-particles (data from Schwarm et al. 2008, 2009c for banteng *Bos javanicus* on the low intake feeding regime), the dry matter gut fill as calculated by equation 5 (see methods) would be 5.2 or 5.9 kg, respectively, depending on whether the small or the large particle retention time is used for the calculation – a difference of about 13 %.

In macropods, a differential passage of different sized particles has been suspected (Hume 1999). In tammar wallabies, the proportion of small particles in the forestomach decreases with time since feeding (Lentle et al. 2007), and in both tammar wallabies and red-necked wallabies (*Macropus rufogriseus*), a finely-ground diet increased the material present in the caecum and colon, suggesting that fine particles pass through the forestomach quicker and accumulate in the hindgut (Munn et al. 2006; Munn et al. 2007). These findings could suggest a selective expulsion of fine particles from the macropod forestomach. Empirical evidence from trials in which different-sized particle markers were fed contradicts this assumption (Schwarm et al. 2009b), but this might have been due to the comparatively large particle-size classes used (1 cm and 2 mm particles). Therefore, when validating the Holleman & White-approach to estimate gut fill in wallabies, we also compared the retention times of two differently-sized small-particle markers and their subsequent effect on estimates of gut fill.

Materials and Methods

Animals and experiment

Seven adult female tammar wallabies (age 5-8 years; body mass $5.2 \pm 0.2 \text{ kg}$) were housed in concrete cages (230 cm high \times 125 cm wide \times 235 cm long) on wood shavings. Animals were maintained under a 12 hour light:12 hour dark cycle. Chopped lucerne hay (Kensington Produce, Sydney, Australia) and water were available *ad libitum*. Eight days prior to the feeding trial, animals were taken from their meadow enclosure, separated and housed in individual cages as above. For the duration of the feeding trial, wood shavings were taken away to facilitate easy removal and non-contamination of the feces. During the trial, samples of feed offered and total feces were collected and stored frozen at -24°C . Wallabies were

weighed at the beginning and again at the end of the experimental trial. Food intake and fecal output were measured daily. Each day the wallabies were offered 1.5 times the previous day's food intake.

The mean retention time of solutes and particles in the gastrointestinal tract was measured using three inert markers. The solute marker used was cobalt-ethylene diaminetetraacetic acid (Co-EDTA). Particles were marked with either chromium (Cr) or cerium (Ce) mordanted to plant cell walls (CW) according to Udén et al. (1980). Cell walls were prepared from chopped lucerne hay dried at 60°C, ground through a 1 mm mesh and treated with neutral detergent (Van Soest et al. 1991) and wet-sieved through a series of Endicott (London, England) screens. Particles that passed through a 1 mm screen but trapped on a 500 µm screen were retained for mordanting with Cr; particles that passed through a 500 µm screen but were trapped on a 100 µm screen were retained for mordanting with Ce.

Seven wallabies were offered a single dose of 1.0 g Cr-CW and 0.5 g Co-EDTA between 8-10 am on the first day of the trial. Due to expected problems with marker intake based on the observations during the acclimation period, only four of these seven wallabies were additionally offered Ce-CW (two at a dose of 1.0 g and two at 0.8 g). The complete dose was mixed with unsweetened apple juice and spread on a quarter slice of stale fruit bread, which was consumed by each animal within ten minutes. After dosing, feces were collected at 2-hour intervals for 24 hours, followed by 4-hour intervals for 24 hours, then 6-hour intervals for 24 hours, 12-hour intervals for 24 hours and finally a 24 hour collection (total 120 hours); a subsample of app. 10% of the total fecal interval output was stored frozen until analysis.

After the experimental trial (5 days), all animals were euthanized by injection of sodium pentobarbitone (162 mg/kg) via the lateral tail vein. Animals were euthanized between 8-10 a.m. after the morning feed and were immediately dissected. Dissection was via a ventral incision and the entire gastrointestinal tract was removed and ligated at the junction of each major section to minimize mixing between compartments. The foregut was separated cranially at the esophageal junction and caudally at the pyloric sphincter and then the small intestine was tied caudally at the ileocecal junction. The cecum was tied from the proximal colon, also at the ileocecal junction, while the proximal colon was tied and distinguished from the distal colon at the point where fecal pellets formed (Dellow and Hume 1982b). The entire

gastrointestinal tract was then cleaned of mesentery, connective tissue and fat. Each gut section (foregut, small intestine, cecum, proximal colon and distal colon) was then separated and rinsed, blotted dry and weighed. The sections were then emptied and samples of the contents of each gut section were obtained and stored frozen (-24°C). After emptying, gut sections were re-rinsed, blotted dry and reweighed to determine digesta load (g; wet mass) and organ empty wet mass (g). Samples of gut contents were dried in a 60°C oven for several days to obtain gastrointestinal DM content (DCM).

Analysis of samples

Sub-samples of chopped lucerne hay offered, and wet gut-contents together with the feces collected during the trial were thawed and dried at 60°C for several days. Dried feces were ground through a 1 mm mesh using a hammer mill (Glen Creston, Stanmore) in preparation for further analysis. Fecal samples were analyzed for Co, Cr and Ce concentrations using an Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES; Vista AX, Varian; California, USA) after preparation by microwave-acid-assisted digestion performed using a Milestone 1200 mega microwave digester (Shelton, Connecticut, USA). Sub-samples of ground, dry fecal sample were placed in a tetrafluormethaxil (TFM) vessel and 10 ml of 70% nitric acid was added. The digestion program was as follows: 250 W/2 min; 0 W/2 min; 250 W/5 min; 400 W/5 min; then 600 W/5 min. After cooling, the contents were completely transferred to a specimen container and diluted to 25 ml with de-ionized water.

Calculations

Apparent digestibility aD, in %) of dry matter (DM) was calculated as:

$$\frac{\text{intake} - \text{faecal output}}{\text{intake}} \times 100 \quad (1);$$

where intake and fecal output are in g day⁻¹ (Robbins 1993).

The MRT for the whole gastrointestinal tract (MRT GIT) was calculated according to Thielemans *et al.* (1978) as

$$\text{MRT GIT} = \frac{\sum t_i C_i dt_i}{\sum C_i dt_i}$$

(2),

with C_i = marker concentration in the faecal samples from the interval represented by time t_i (hours after marker administration) and dt_i = the interval (hours) of the respective sample

$$dt_i = \frac{(t_{i+1}-t_i)+(t_i-t_{i-1})}{2} \quad (3).$$

The indigestible dry matter **gut** content (indDMC, g) and the total dry matter **gut** content (DMC, g) were calculated according to Holleman & White (1989):

$$\text{indDMC} = F * \text{MRT} \quad (4),$$

with F (faeces output, kg DM/h) = total daily faeces output/24 and with MRT = mean particle retention time through the whole digestive tract (h). In order to yield DMC, the proportion of digestible DMC must be added to the indDMC. This is done making basic assumptions on the occurrence of digestion with MRT :

$$\text{DMC}_{\text{lin}} = \text{indDMC} + ((\text{indDMC} * (\text{aD DM}/100))/(2(1 - (\text{aD DM}/100)))) \quad (5),$$

assuming linear absorption of ingested food with time spent in the tract (note that this does not mean linear absorption along the digestive tract) and;

$$\text{DMC}_{\text{exp}} = (\text{indDMC} - ((\text{indDMC} / (1 - (\text{aD DM}/100)))) / (\ln(1 - (\text{aD DM}/100))) \quad (6),$$

assuming exponential absorption of ingested food with time spent in the tract (note again that this does not mean exponential absorption along the digestive tract); with aD DM = apparent dry matter digestibility, and see below for explanation of bold typeset **indDMC**.

In a second attempt to calculate DMC in a more realistic manner, the portion of digestible DMC was calculated not based on total MRT (because in the final stages of MRT digesta rests in the distal colon and rectum and no further digestion takes place). Thus we estimated the ‘corrected’ DMC ($\text{DMC}_{(c)}$) based on both, total tract MRT (for the indigestible portion of DMC) and on total tract MRT minus MRT of material held in the distal colon (for the digestible portion of DMC). The latter information was taken from Dellow (1982) who found that MRT in the distal colon of tammar wallabies fed chopped lucerne hay ad libitum was 21.3% of total tract MRT . Therefore, in equations (5) and (6), the **indDMC** set in bold was

calculated by multiplying daily faecal output with the estimated MRT from the cardia to the proximal colon (i.e. total MRT – estimated distal colon MRT).

Data were analysed by Pearson's correlation coefficient (R) and by paired t-test using PASW 18.0 (SPSS Inc., Chicago, IL). Results are presented as means \pm standard deviation.

Comparative data on tammar wallabies from different sources (Dellow 1982; Dellow and Hume 1982b; a; Lentle et al. 1998; Munn et al. 2006) were included in the results tables 1 and 2.

Results

Wallabies maintained body mass throughout the experimental trial, with a mean difference in body mass between the start and end of the experiment of 0.07 ± 0.10 kg ($p=0.094$). At $34 \text{ g kg}^{-0.75} \text{ d}^{-1}$, the relative dry matter intake (rDMI) of the tammars was similar to that of animals also fed chopped lucerne in two other studies (Dellow 1982; Dellow and Hume 1982b) but lower than the $53 \text{ g kg}^{-0.75} \text{ d}^{-1}$ measured in another group of animals (Dellow and Hume 1982a) (Table 1). aDDM was, at 59%, very similar to the aD of organic matter of 60% measured by Dellow & Hume (1982b) on a similar rDMI.

The excretion of the passage markers followed the biphasic pattern for solutes and particles described by Dellow (1982) (Fig. 1). $\text{MRT}_{\text{particlesGIT}}$ (measured by Cr) was significantly longer than $\text{MRT}_{\text{soluteGIT}}$ (mean difference 9.8 ± 3.0 h, $p<0.0001$); both measures were similar to the 50% marker excretion times reported by Dellow (1982) in animals on a similar intake level (Table 1). In the four animals that received both particle markers, there was no significant difference between the $\text{MRT}_{\text{particlesGIT}}$ of the small and the medium-sized particles (mean difference 0.5 ± 2.5 h, $p=0.733$; Fig. 1); therefore, gut content estimates are given in the following for the results of the Cr-CW only. There was no significant relationship between either body mass or the relative dry matter intake with aD DM (BM-aD DM $R=0.11$, $p=0.812$; rDMI-aD DM $R=-0.24$, $p=0.611$), any MRT measurement (BM- $\text{MRT}_{\text{particles}}$ $R=-0.26$, $p=0.573$; BM- $\text{MRT}_{\text{solute}}$ $R=-0.09$, $p=0.853$; rDMI- $\text{MRT}_{\text{particles}}$ $R=-0.67$, $p=0.100$; BM- $\text{MRT}_{\text{solute}}$ $R=-0.50$, $p=0.255$), or the ratio of $\text{MRT}_{\text{particles}}/\text{MRT}_{\text{solute}}$ (BM-SF $R=-0.37$, $p=0.416$; rDMI-SF $R=-0.32$, $p=0.492$).

The wet matter contents (WCM) of the individual gut sections of the tammar wallabies of this study were very similar to those recorded in animals on the same diet and food intake level reported by Dellow & Hume (1982b), and for animals on a natural diet (Munn et al. 2006), but were lower than that of free-ranging tammar wallabies (Lentle et al. 1998) (Table 2). The proportion of contents of individual gut sections relative to overall gut contents was similar in all animal groups investigated (Table 2), with the forestomach complex containing about three quarters of the total gut contents. The dry matter concentration of the gut contents was lowest in the small intestine and increased from the caecum to the proximal and distal colon, respectively (Table 3). There was no significant relationship of either body mass or the relative dry matter intake with the WMC (BM-WMC $R=-0.16$, $p=0.735$; rDMI-WMC $R=-0.67$, $p=0.103$), DMC (BM-DMC $R=-0.33$, $p=0.469$; rDMI-DMC $R=-0.54$, $p=0.213$) or water gut-content (BM-water $R=-0.08$, $p=0.859$; rDMI-water $R=-0.58$, $p=0.176$). In contrast, MRTs were highly correlated with WMC (MRT Cr $R=0.96$, $p<0.0001$; Fig. 2a) but not to DMC (MRT Cr $R=0.46$, $p=0.294$; Fig. 2b). WMC was not correlated with dry matter gut content (DMC) as measured by dissection ($R=0.36$, $p=0.432$; Fig. 3a); in contrast, WMC was highly correlated with the water gut-content ($R=0.89$, $p=0.007$).

Whether calculated using the linear or exponential equation of Holleman & White (1989), the calculated DMC was, at 83.3 ± 12.0 and 78.1 ± 10.6 g, respectively, distinctively higher than that measured directly (65.0 ± 6.9 g) (Table 4). There was no correlation between the calculated DMC and the DMC as measured by dissection (DMC_{lin}-DMC_{dissection}: $R=0.24$, $p=0.610$; DMC_{exp}-DMC_{dissection}: $R=0.26$, $p=0.581$), but the calculated DMC was significantly correlated with the WMC as measured by dissection (DMC_{lin}-WMC: $R=0.82$, $p=0.024$, Fig. 3b; DMC_{exp}-WMC: $R=0.80$, $p=0.029$). A linear regression through the origin for WMC and DMC_{dissection} (Fig. 3a) and DMC_{lin} (Fig. 3b) yielded slopes of 0.17 and 0.22, respectively, which corresponds to assumed DM concentrations of total gut contents of 17% (as actually measured, Table 2) or 22%. In other words, calculated and measured DMC would have corresponded if, rather than an average 17% of dry matter in the total gut contents, 22% would have been determined when drying the digesta samples. Combining the data on digestibility for ad libitum chopped lucerne hay from Dellow & Hume (1982b) with the intake and retention data from Dellow (1982) yields a calculated DMC of 83 g and 77 g for the linear and the exponential equation, respectively, which is similar to the 83 g and 78 g of this study. The measured WMC in Dellow & Hume (1982b) was, at 367 g, similar to the

WMC in this study (383 g). Thus, in theory, the calculated DM concentration of the total gut contents in the study by Dellow & Hume should have been 22.5% - again similar to the calculated 22% of this study.

When correcting the DMC by calculating the digestible portion for the MRT that excluded the distal colon, the resulting corrected DMC ($DMC_{(c)}$) was - as expected - lower (Table 4) and, in the case of the exponential equation, even lower than the actually measured one. Again, there was no correlation between the calculated $DMC_{(c)}$ and DMC as measured by dissection ($DMC_{lin(c)}-DMC_{dissection}$: $R=0.25$, $p=0.591$; $DMC_{exp(c)}-DMC_{dissection}$: $R=0.20$, $p=0.668$), but the calculated $DMC_{(c)}$ was significantly correlated with the WMC as measured by dissection ($DMC_{lin(c)}-WMC$: $R=0.82$, $p=0.024$, Fig. 3b; $DMC_{exp}-WMC$: $R=0.80$, $p=0.029$).

Discussion

Lack of particle-size differentiation in the macropod forestomach

The results of this study corroborate previous evidence (Langer et al. 1980; Schwarm et al. 2009b) that, in contrast to common expectations (Hume 1999, p. 240; Munn and Dawson 2006; Lentle et al. 2007; Munn et al. 2007), the forestomach of macropods does not appear to differentiate the passage of differently-sized particles. On the one hand, it has been suggested that, similar to the large intestine of rabbits, the macropod forestomach eliminates large particles faster than small particles (Hume 1999, p. 240). The benefit of this would be that those particles that are more difficult to digest due to an unfavourable surface:volume-ratio are cleared from the gastrointestinal tract sooner. Evidence for such an excretion pattern in foregut fermenters is, however, limited to some individual hippopotamuses (Clauss et al. 2004; Schwarm et al. 2008). On the other hand, an assumed similarity in the function of the macropod forestomach with the haustrated colon of hindgut fermenters, would lead to the conclusion that in macropods very small particles should be eliminated faster from the forestomach as compared with large particles. This reflection is based on several observations. In studies with macropods, the finding that solute markers pass the gastrointestinal tract faster than particle markers is pervasive (Warner 1981; Dellow 1982; Bridie et al. 1994; Wallis 1994; Munn and Dawson 2006; Schwarm et al. 2009b), and the study of Dellow (1982) confirmed that this is due to a differential passage of solute and particles through the forestomach. Such a differential passage of solutes and particles has also been documented in hippopotamuses (Clauss et al. 2004; Schwarm et al. 2008). In rabbits,

which have a haustrated colon, solute and particle markers are also eliminated separately – but with an inverse pattern: in rabbits, the solute marker is retained for a longer time (Franz et al. 2011 and references therein). This finding is linked to a retrograde (orad) peristalsis of the colonic haustrae (Ehrlein et al. 1983). This process has been shown to separate very fine particles – i.e., bacteria – from the digesta and to concentrate them in the caecum, from where they are excreted as cecotrophs for re-ingestion (reviewed in Franz et al. 2011), but commonly used particle markers do not follow this pattern. The finding of an accelerated solute marker excretion from the forestomach of macropods suggests a propulsive (aborad) peristalsis of the forestomach haustrae as demonstrated in some species (Dellow 1979; Richardson and Wyburn 1983; 1988; Wyburn and Richardson 1989). One function of this mechanism, similar to the rabbit, is most likely an intensified ‘harvest’ of bacteria from the forestomach contents (Müller et al. 2011). Studies using novel markers, such as labelled bacteria (Takahashi and Sakaguchi 2006), in parallel with solute and particle markers, would be required to finally corroborate this concept. However, because neither small nor large forage particles are **selectively** retained in the macropod forestomach (Schwarm et al. 2009b; this study), no sorting mechanism, and no relief of the general intake-limiting condition of foregut fermentation (Clauss et al. 2010a), can be assumed for macropods.

Estimating gut fill

There is an evident logic to the Holleman & White-approach to estimate indigestible and total dry matter contents of the digestive tract, and the approach was empirically corroborated based on a similarly small sample size ($n=5$ sheep as compared to the $n=7$ tammar wallabies) in the original study (Holleman and White 1989). However, we found that the calculated DMC deviated, depending on the method of calculation, by between -23 ± 14 % for $DMC_{exp(c)}$ to 29 ± 22 % for DMC_{lin} from the measured DMC, and, even worse, did not correlate significantly with the measured DMC. This represents a conceptual problem that warrants explanation if the Holleman & White-approach to estimating gut fill is to be used in further studies.

Several factors could help to explain the discrepancy we found between the measured and the **calculated** gut fill in tammar wallabies. In theory, an underestimation of the measured DMC may be expected, because the **calculated** DMC represents only the indigestible and apparently digestible material in the gut, and excludes endogenous secretions into the gastrointestinal

tract and microbial fauna; endogenous secretions and microbes are present at dissection but are at least partly absorbed or digested prior to faecal elimination, and hence do not contribute to the measure of apparent digestibility, a key parameter to the gut fill model. Holleman and White (1989) recognised this drawback in their original work, and their model underestimated measured gut fill in four out of the five sheep (Holleman and White 1989). However, in our case most of the **calculated** DMC measurements were higher, not lower, than the measured values, which is contrary to Holleman and Whites (1989) finding for sheep.

One possible explanation for the discrepancy we found between the **calculated** and the measured DMC of tammar wallabies could be a consequence of the methods used to measure DMC post-mortem, due to irregularities in the determination of the gut contents' DM concentration. In our case, DM concentrations were determined by drying at 60°C, necessary for fibre analysis for a related study; strictly, DM contents analyses should be performed at 103°C to ensure complete drying (AOAC 1997). Less than **thorough** drying could therefore explain some of the **differences** we observed between the **measured** and **calculated** gut fills. The linear regressions of WMC and DMC suggest that a slight underestimation of the DM concentration of gut contents had occurred (Fig. 3ab), so incomplete drying appears to be a plausible explanation for overestimating measured fill (but notice also that the relative pattern of DM concentration along the digestive tract was as expected for this species; Table 3). Consequently, more complete DM determination by drying at the adequate temperature may be of paramount importance for future studies. Nonetheless, if such an irregularity in the determination of the DM concentration had occurred systematically in our study, one would expect the measured DMCs values to correlate significantly with the **calculated** DMCs values, but this was not the case, indicating that either irregularities in DM determination were not systematic (a consideration not compatible with the systematic pattern of DM concentration in the individual gut segments; Table 3), or that other reasons for the discrepancy between our measured and modelled gut fill must be sought.

Another possible explanation for the observed discrepancy we found between the measured and the **calculated** DMC of tammar wallabies is related to a fundamental precondition for the Holleman & White-approach – the assumption of steady-state conditions. Holleman & White (1989) stated repeatedly that steady-state conditions must be given, due to the integrative nature of the digestibility coefficient and the retention time measurements, which must be

performed over several days or more in medium- and large-sized herbivores (from a few kg upwards) that show considerable inter-meal digesta mixing. Thus, average feed intakes, digestibilities and MRTs are derived from, and representative for, a period of up to several days, but the results of post-mortem quantification of gut contents are representative only for one particular point in time. In particular, if the food intake prior to dissection was lower, or had even ceased for some time before death, the measured gut fill will not correspond to that calculated for an animal integrating data over an entire feed-trial period. Several indications suggest that this may have occurred in our case. For example, if we compare the forestomach gut-fill for our animal with those from Lentle (2007), who sacrificed tammar wallabies after increasing periods of fasting, we can suspect that our animals may not have fed for an average of 6 ± 4 h prior to euthanasia (Fig. 4). Further, because these estimated fasting periods differed for individuals, one could suspect that this effect accounts for the lack of correlation we found between the calculated and the measured DMCs. As such, we recommend that future studies carefully monitor and define food intake prior to animal euthanasia and subsequent dissection.

Additionally, other data collected here suggests that our animals may have deviated from steady-state conditions immediately before death, which could have influenced the results. For example, some individuals had low food intakes during the last trial day (day 5). When expressed as the difference between feed intake on day 5 and the average intake over the whole five days, we found a negative correlation between the calculated DMC_{lin} and measured DMC (Fig. 5), indicating that in those animals that ate unusually small amounts on day 5 of their trial (the last day prior to dissection) compared with other days, DMC was notably lower than that calculated according to data collected over the entire five-day trial. Once again, future studies should take care to achieve steady intakes over the entire trial, and should ensure that intakes on the final day immediately prior to dissection should be representative of intakes observed across the entire period.

An interesting finding of this study was that the calculated DMC, which could be, due to the reflections listed above, considered as an integrative measure over a certain period, correlated with the actually measured wet matter content (WMC). This could mean that WMC actually represents a measure that is less affected by short-term changes in food intake than DMC. This interpretation is supported by the fact that WMC correlated positively with the feed

retention times measured in the experiment (Fig. 2a). Whether this means that the wallabies compensated for a low DMI by increased water intake, or whether saliva or other fluid secretions account for this compensation, remains to be investigated.

Conclusion

In macropods, particle markers within the size range of 100 μm up to 1 cm do not indicate a selective retention or excretion of small or large particles. Additional studies are necessary to assess whether particularly small particles, such as bacteria or other particles <100 μm , are flushed aborad with the fluid phase, as fluids are known to pass through the forestomach faster than the digesta particles in macropods (Fig. 1). Overall, the Holleman & White-approach to estimating gut fill in live animals appears logical and valid, but testing it requires a rigorous protocol to standardize food intake prior to dissection, as well as during sample drying. The correlation of the dry matter gut content as calculated using the Holleman & White-approach with the measured wet gut contents, which were also highly correlated to digesta retention times, suggests that estimation of gut fill via this approach can yield valuable insights into digestive adaptations. Given the results presented in the original work (Holleman and White 1989) and the technical problems associated with determining digesta retention for that part of the colon where no further digestion takes place, the DMC_{lin} appears to be, so far, the most promising parameter to calculate dry matter gut fill.

Acknowledgments

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Table 1. Mean (\pm SD) body mass, dry matter intake, digestibility and measures of digesta retention in tammar wallabies (*Macropus eugenii*) in this study (n=7) and other studies.

	This study	(Dellow and Hume 1982a)	(Dellow 1982)	(Dellow and Hume 1982b)
Body mass (kg)	5.2 \pm 0.4	5.0	4.8	4.5
DMI (g d ⁻¹)	115 \pm 19	179	120	98
rDMI (g kg ^{-0.75} d ⁻¹)	34 \pm 5	53	37	32
aD DM (%)	59 \pm 3	55		60 ^a
MRT Cr (h)	25 \pm 6 (27 \pm 8) ^b	-	24 ^c	-
MRT Ce (h)	27 \pm 6 ^b	-	-	-
MRT Co (h)	16 \pm 6	-	15 ^c	-
SF	1.65 \pm 0.19	-	1.57	-

DMI dry matter intake; rDMI relative dry matter intake; aD DM apparent digestibility of dry matter; MRT mean retention time; Cr chromium (mordanted to particles between 500-1000 μ m); Ce cerium (mordanted to particles between 100-500 μ m); Co cobalt (solute marker); SF selectivity factor (the ratio of MRT Cr/MRT Co)

^a apparent digestibility of organic matter

^b data for animals that also received the Ce marker (n=4)

^c 50% marker excretion time for ruthenium phenantroline as a particle and Cr-EDTA as a solute marker

Table 2. Mean (\pm SD) wet matter gut contents (WMC), the proportion of WMC in individual gut segments, dry matter concentration, gut water content, and dry matter gut contents (DMC) as measured by dissection in tammar wallabies (*Macropus eugenii*) fed either chopped lucerne ad libitum (this study; Dellow and Hume 1982b) or on natural diets (Lentle et al. 1998; Munn et al. 2006).

	----- WMC (g) -----				----- WMC % of total contents -----			
	(Dellow and Hume		(Munn et al. 2006)		(Dellow and Hume 1982b)		(Munn et al. 2006)	
	this study	1982b)	(Lentle et al. 1998)		this study	(Dellow and Hume 1982b)	(Lentle et al. 1998)	
Body mass (kg)	5.2 \pm 0.4	4.5	4.5	4.9	-	-	-	-
rDMI (g kg ^{-0.75} d ⁻¹)	34 \pm 5	32	-	-	-	-	-	-
Stomach	279 \pm 29	279	425	293	73 \pm 3	76	74	79
Small intestine	44 \pm 12	33	58	28	11 \pm 3	9	10	7
Caecum	14 \pm 6	-	24	-	4 \pm 2	-	4	-
Proximal colon	21 \pm 6	-	-	-	6 \pm 2	-	-	-
Distal colon	25 \pm 6	-	-	26	7 \pm 2	-	-	7
Whole colon	46 \pm 2	-	66	-	12 \pm 1		12	-
Whole hindgut	60 \pm 5	55	91	78	16 \pm 2	15	16	21
Total	383 \pm 29	367	574	373	100	100	100	100

Table 3. Mean (\pm SD) dry matter concentration, gut water content, dry matter gut contents (DMC) as measured by dissection in tammar wallabies (*Macropus eugenii*) fed chopped lucerne ad libitum.

	DM %	Water (g)	DMC (g)
Stomach	16.6 \pm 2.3	233 \pm 26	46.2 \pm 7.0
Small intestine	11.6 \pm 1.1	39 \pm 11	5.1 \pm 1.4
Caecum	15.9 \pm 1.4	12 \pm 6	2.2 \pm 0.9
Proximal colon	17.4 \pm 0.7	17 \pm 5	3.6 \pm 1.0
Distal colon	31.4 \pm 3.8	17 \pm 4	7.9 \pm 2.3
Whole colon	25.1 \pm 3.0	34 \pm 2	11.5 \pm 1.5
Whole hindgut	23.1 \pm 2.9	46 \pm 6	13.7 \pm 0.8
Total	17.0 \pm 1.8	318 \pm 27	65.0 \pm 6.9

Table 4. Mean (\pm SD) indigestible dry matter **gut** content (indDMC) and dry matter **gut** content (DMC) of the gastrointestinal tract in tammar wallabies (*Macropus eugenii*) fed chopped lucerne ad libitum using different ways of measurement/estimation

	indDMC (g)	DMC _{lin} (g)	DMC _{exp} (g)
measured by dissection	-	65.0 \pm 6.9	
equations by Holleman & White (1989)	48.4 \pm 5.1	83.3 \pm 12.0	78.1 \pm 10.6
equations by Holleman & White (1989) with correction for digesta retention in the distal colon		75.9 \pm 10.5	49.9 \pm 7.9
_{lin} calculated using the linear equation (equation 5 in methods)			
_{exp} calculated using the exponential equation (equation 6 in methods)			

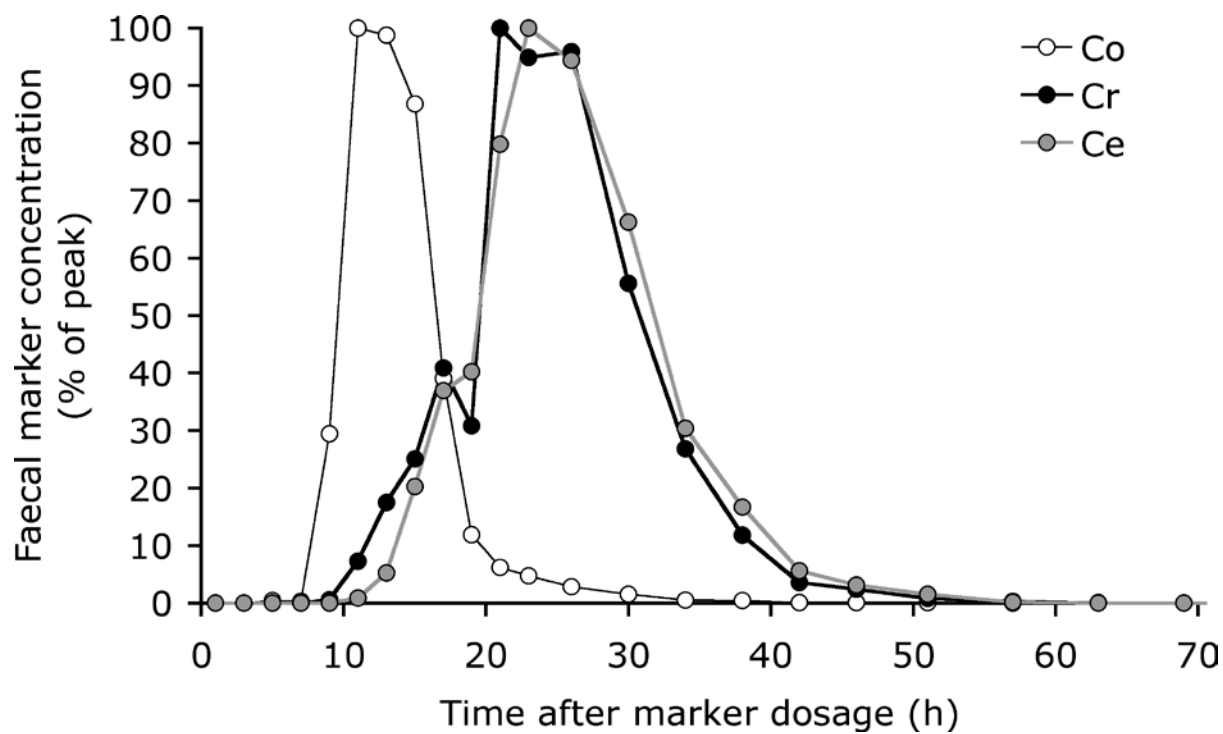


Figure 1. Marker excretion pattern in a tammar wallaby (*Macropus eugenii*) fed chopped lucerne hay ad libitum, given cobalt (Co) EDTA as a solute, and chromium-mordanted fibre (500-1000 μm) and cerium (Ce) mordanted fibre (100-500 μm).

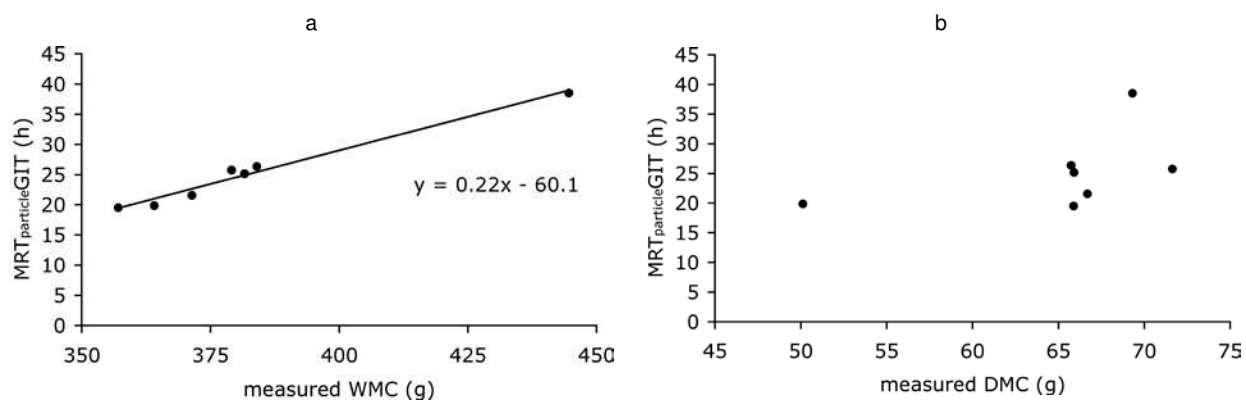


Figure 2. Relationship between particle mean retention time (MRT) in the gastrointestinal tract (GIT) of tammar wallabies (*Macropus eugenii*) on chopped lucerne ad libitum and a) the wet matter contents (WMC) of the GIT and b) the dry matter contents (DMC) of the GIT. Note that only the relationship with WMC was significant (see results).

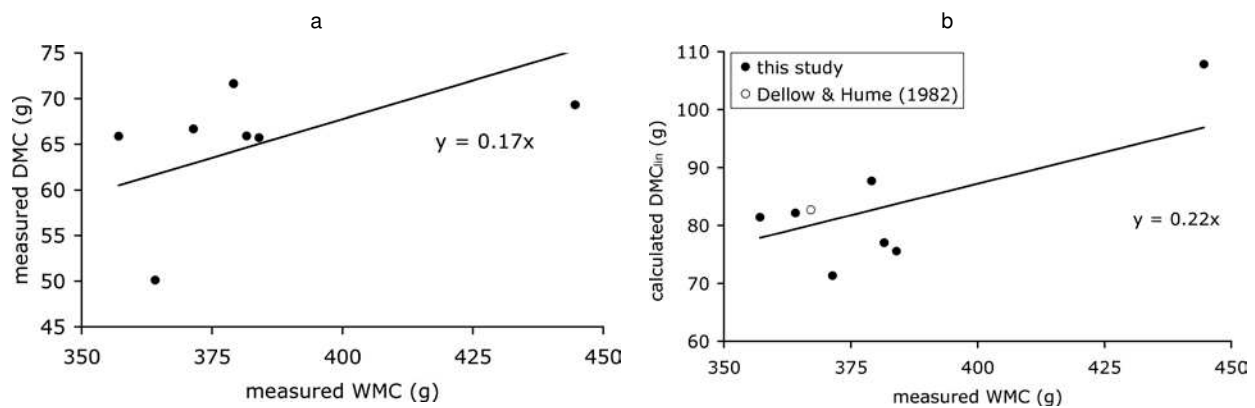


Figure 3. Relationship between the wet matter contents (WMC) of the gastrointestinal tract of tammar wallabies (*Macropus eugenii*) fed chopped lucerne ad libitum and a) the dry matter contents (DMC) as measured by dissection and b) the DMC as calculated from data on intake, digestibility and digesta retention using the linear Holleman and White (1989) equation. Note that linear regression suggests a dry matter concentration of 17% in a) and 22% in b). The open data point in b) was gathered on tammar wallabies on chopped lucerne ad libitum (Dellow 1982; Dellow and Hume 1982b).

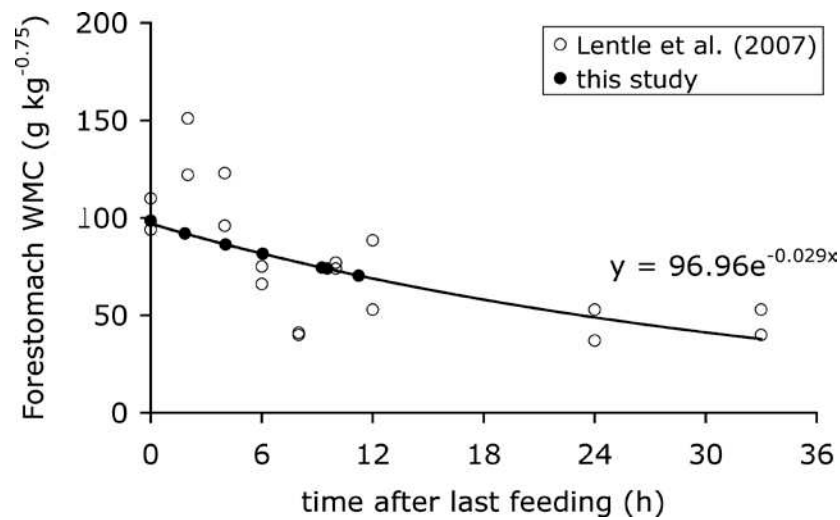


Figure 4. Relationship between the relative wet content mass in the forestomach of tammar wallabies (*Macropus eugenii*) from Lentle et al. (2007; open symbols, data read from graph) and the corresponding results from this study (solid symbols) that suggest that animals of this study could have had varying periods of suspended food intake (regardless of ad libitum offering of food).

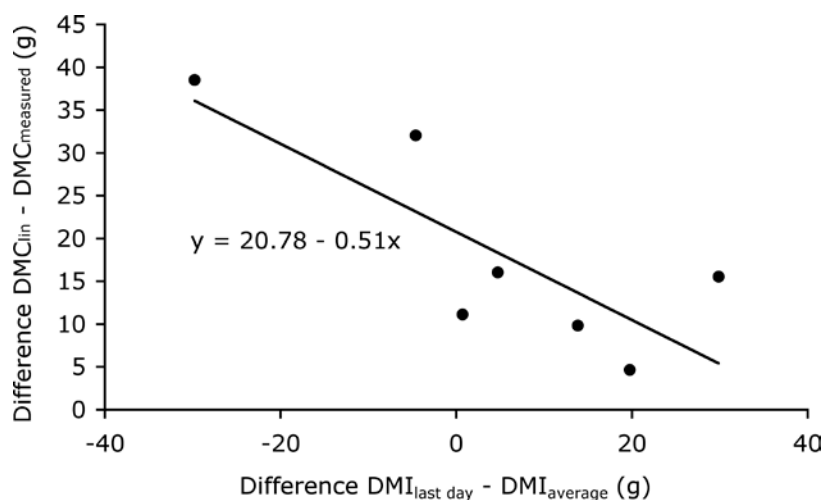


Figure 5. Relationship between the difference in dry matter intake (DMI) between the last day prior to dissection and the average of the trial period and the difference between the dry matter content (DMC) as calculated by the Holleman & White (1989) equation (DMC_{lin}) and the actually measured DMC. The relationship was significant ($R=-0.80$, $p=0.030$), indicating that the discrepancy between the two DMC values was particularly high in animals in which food intake during the last trial day was particularly low.